Journal of Pediatric Genetics 2 (2012) 99–102
DOI 10.3233/PGE-2012-017
IOS Press

SMN1 gene copy number analyses for SMA healthy carriers in Italian population

Alessandra Patitucci[a], Angela Magariello[a], Carmine Ungaro[a], Maria Muglia[a], Francesca L. Conforti[a], Anna L. Gabriele[a], Luigi Citrigno[a,b], William Sproviero[a,c] and Rosalucia Mazzei[a,*]
[a]*Institute of Neurological Sciences, National Research Council, Mangone, Cosenza, Italy*
[b]*Department of Neuroscience, Psychiatric and Anesthesiological Sciences, University of Messina, Messina, Italy*
[c]*Department of Neurology, University "Magna Graecia", Catanzaro, Italy*

Received 5 April 2011
Revised 13 July 2011
Accepted 29 July 2011

Abstract. The routine molecular test for spinal muscular atrophy (SMA) diagnosis is based on the detection of a homozygous deletion of exons 7 and 8 of the telomeric copy of the survival motor neuron gene (*SMN1*). The presence of the centromeric copy of the *SMN* gene (*SMN2*) does not allow the detection of the hemizygous absence of the *SMN1* gene, which characterizes the disease carriers. The demand for a quantitative *SMN1* test is permanently growing because there is a high incidence of carriers. The disease is severe and to date there are no effective pharmacological treatments. Here, we present a non-radioactive assay based on real time quantitative polymerase chain reaction. We analyzed eight SMA patients, 14 SMA relatives and 50 health individuals from Southern Italy by real time quantitative method in order to identify haploid deletion occurring in SMA carriers. *SMN1* copy number was determined by the comparative threshold cycle method (ΔΔCt). The results confirmed the deletion in all homozygous patients and permitted an evaluation of the number of alleles in the healthy carriers. This method is fast, reproducible, and enables us to discriminate carriers from healthy homozygous, which is impossible with normal techniques.

Keywords: SMA, SMN1, real time PCR, quantitative assay

1. Introduction

Autosomal recessive spinal muscular atrophy (SMA) is a neuromuscular disorder caused by the degeneration and loss of the alpha-motor neurons and spinal cord anterior horn cells, which results in symmetric proximal muscle weakness. Patients with SMA were classified into four groups according to age at onset and maximum motor function [1]. Type I SMA is the most severe form (Werding-Hoffman disease; MIM # 253300) with clinical onset before 6 mo of age; type II SMA (MIM # 253550) is the intermediate form with onset before 18 mo of age; type III SMA (Kugelberg-Welander disease; MIM # 253400) is a mild form with onset after the age of 18 mo; and type IV SMA is the adult onset form. The first three forms and most patients affected by SMA IV are due to the homozygous absence of the survival motor neuron gene (*SMN1*; MIM # 600354) located in a complex region of chromosome 5q13 [2]: this region contains a homologous gene (*SMN2*; MIM # 601627) differing in only five nucleotide exchanges, two of them located within exon 7 and 8 respectively [3]. The base-pair exchange in exon 7 permits distinction between the *SMN1* and *SMN2* genes and this is used to detect homozygous deletion

*Corresponding author: Rosalucia Mazzei, Institute of Neurological Sciences, National Research Council, Loc. Burga – 87050, Mangone, Cosenza, Italy. Tel.: +39 0984 9801217; Fax: +39 0984 969306; E-mail: r.mazzei@isn.cnr.it.

of *SMN1* [4]. Childhood-onset SMA affects about 1:10,000 newborns, with a carrier frequency of 1:50 [5]. Because of this high frequency in healthy population, it can be very important to identify carriers of heterozygous deletions of the *SMN1* gene. In order to assess the gene copy number of the *SMN1* gene, we performed real time polymerase chain reaction (PCR) using the comparative method of ΔΔCt. This method allowed us to discriminate *SMN1* from *SMN2*, because we used a probe able to anneal to *SMN1* only. We screened eight patients from six families of SMA patients including the probands, their relatives and 50 normal controls.

2. Materials and methods

2.1. Patients

We investigated six probands and 14 relatives (12 parents and two sibs) and 50 normal controls. SMA patients were previously diagnosed by amplification of exon 7 followed by digestion with DraI [6] and by denaturing high-performance liquid chromatography analysis [7].

2.2. DNA extraction

High molecular weight DNA was extracted from peripheral blood using a salting out method [8]. The optical density at 260/280 method using a photometer was used to determine the appropriate DNA concentration. DNA was diluted in 10mM Tris-HCl containing 1mM EDTA buffer to a final concentration of 6 ng/μL, stored at 4 °C and mixed immediately before use.

2.3. Primers and probe

TaqMan^{MGB} probe and primers were designed using the Primer Express software (Applied Biosystems, Weiterstadt) following the criteria indicated in the program. In order to amplify *SMN1* and not *SMN2*, we chose the probe showing the discriminating base in the middle of the sequence. The probe uses minor groove binder (MGB) technology (Applied Biosystems); it has a 5'-fluor label (6 FAM) as a reporter and a non-fluorescent quencher. The MGB group is attached at the 3' end with the quencher dye; when the probe hybridizes, the MGB group stabilizes the annealing by folding into the minor groove of the DNA duplex created between probe and the target sequence. This stabilization is much more effective when the duplexes match perfectly: thermal stability increases so that we can use TaqMan^{MGB} probes that are very short compared to classical TaqMan probes. Probe and primers sequences are listed in Table 1. We used ribonuclease P (RNaseP) (Applied Biosystems) as housekeeping gene.

2.4. Quantitative analysis

PCR was carried out in a 384-well optical plate closed by adhesive cover using ABI Prism 7900 Sequence Detection System (Applied Biosystems) with a final reaction volume of 20 μL. All samples, prepared from the same master mix, were run in triplicate. For the SMN assay, each well contained 5.4 ng of DNA, 2x TaqMan PCR master mix (Applied Biosystems), 200 nM probe SMN1-ex7, 300 nM each primer and high-pressure liquid chromatography pure water. In the TaqMan universal PCR master mix, there is a passive reference dye (ROX) (ROX is a passive reference dye containing 5-carboxy-X-rhodamine) included in the solution that does not engage in the reaction, but provides an internal reference for background fluorescence emission. This is used to normalize the reporter dye signal. RNaseP reference locus assay was prepared in parallel and in the same run; each well contained 5.4 ng of DNA, 2 x PCR master mix, 20x RNaseP and high-pressure liquid chromatography pure water. Thermal cycling conditions included a pre-run of 2 min at 50 °C and 10 min at 95 °C; cycle conditions were 40 cycles at 95 °C for 15 sec and 60 °C for 1 min, according to the TaqMan

Table 1
Probe and primers sequences for survival motor neuron gene 1 -ex7

Survival motor neuron-PROBE MGBNFQ-5'FAM	5'-TGATTTTGTCTGAAACC-3'
Survival motor neuron gene 1 EX 7F	5'-GCTGGCAGACTTACTCCTTAATTTAA-3'
Survival motor neuron gene 1 EX 7R	5'-GTAAAATGTCTTGTGAAACAAAATGC-3'

universal PCR protocol (Applied Biosystems); run time was 1 h and 30 min.

The ΔΔCt method required a healthy control sample (diploid) as calibrator in all amplifications.

2.5. Data analysis

Data evaluation was carried out using the ABI Prism Detection Software and Microsoft Excel. Samples were run in triplicate for *SMN1* and RNaseP. The threshold cycle number (Ct) was determined for all wells. The Ct represents the cycle number (ΔRn) at which the fluorescence emission of the reporter dye passed a fixed threshold. The threshold was automatically set at 10 standard deviations (SD) above the mean baseline emission. For all samples, including the calibrator, the same threshold and baseline were set. The starting gene copy number of the unknown samples was determined applying the following formula described by Livak in 1997: ΔΔCt = [Ct RNaseP (calibrator) − Ct SMN1 − 7 (calibrator)] − [Ct RNaseP (unknown) − Ct SMN1 − 7 (unknown)]. The relative gene copy number was calculated by the expression $2^{-\Delta\Delta Ct}$.

3. Results

Before starting our experiment, we had to optimize primers and probe concentrations. The optimal concentration provides the lowest Ct value and the highest increase in the fluorescence compared to the background. The concentration of the primers should be optimized by spanning an initial concentration range of 50 nM–900 nM, with the concentration of probe in a range from 50 nM–250 nM. Our results showed an optimal concentration of 200 nM and 300 nM for *SMN1*-ex7 probe and each primer, respectively.

To use the comparative method of ΔΔCt, a validation experiment must be run to show that the amplification efficiencies of the target and the endogenous control are approximately equal. The absolute value of the slope of log input amount vs. ΔCt should be < 0.1. The curves obtained passed this test. We then proceeded to analyze samples and calibrator in triplicates and in parallel for *SMN*-ex7 and Rnase P. The copy number of the *SMN1*-ex7 test locus was given by the ΔΔCt formula.

We could clearly distinguish three groups of samples: 1) in homozygous deleted patients there was no amplification with *SMN1*-ex7 probe and primers, while there was amplification with RNaseP and the value derived from comparative ΔΔCt method was 0.005 ± 0.010 (range 0–0.015); 2) in hemizygous samples there was amplification with both *SMN1*-ex7 and RNaseP and the ΔΔCt value was 0.354 ± 0.124 (range 0.23–0.48); 3) in all normal samples the ΔΔCt value was 1.184 ± 0.440 (range 0.75–1.62). No overlap in $2^{-\Delta\Delta Ct}$ values was revealed (Table 2). The reproducibility of this method was checked in 10 independent PCR reactions on 10 different days.

4. Discussion

The diagnosis of SMA is based on the detection of a homozygous deletion of the *SMN1* gene. Both molecular tests available until now (PCR followed by digestion and denaturing high-performance liquid chromatography analysis) are unable to detect hemizygous absence of *SMN1*, which characterizes the healthy SMA carriers. In the present study, we performed an assay based on real time quantitative PCR [9,10–12] using the comparative Ct method (ΔΔCt). By this method, we were able to calculate the gene copy numbers based on a Ct value, rather than standard curve method. Our method overcomes the limit of starting amount of DNA, because a little variation in starting DNA concentration may have a dramatic effect on the amount of PCR product and it can lead to a misinterpretation of data.

The real time PCR is based on the 5' exonuclease activity of the Taq DNA polymerase to cleave a dual labeled fluorogenic probe specifically hybridized to the DNA target. The *SMN1*-ex7 probe was labeled with a

Table 2
ΔΔCt values in examined samples

Subjects	Mean ± SD	Minimum	Maximum
Spinal muscular atrophy patients	0.005 ± 0.010	< 0.015	0.015
Spinal muscular atrophy carriers	0.354 ± 0.124	0.23	0.48
Normal controls	1.184 ± 0.440	0.75	1.62

reporter (FAM) and a quencher (dark) on 5' and 3' end, respectively. After TaqMan probe annealing, the proximity between the reporter and the quencher results in extinction of reporter fluorescence, due to fluorescence resonance energy transfer. During PCR amplification, the probe is cleaved releasing the reporter and resulting in fluorescence emission. The intensity of fluorescence increases at each cycle, proportionally to the amount of target DNA formed [11]. Further advantage of the ΔΔCt comparative method is that, it does not need a standard curve, which can increase throughput because wells no longer need to be used for the standard curve samples. This also eliminates dilution errors made in creating the standard curve samples. All samples were prepared in parallel for *SMN1*-ex7 and RNaseP and tested in triplicate in order to evaluate the degree of accuracy of manual work.

Our data lead us to identify three different groups: SMA patients, healthy controls, and, more importantly, SMA healthy carriers. This finding is very relevant for genetic counseling because of the high incidence of healthy carriers in the population. As for the previously described methods, this application has limitations. It is possible to miss the healthy carriers in two situations: when the healthy carriers have two copies of the *SMN1* gene on the same chromosome (4.8%), as well as in case of intragenic mutations (1.7%) [13,14]. In both situations, the percentage of individuals concerned is very low.

In conclusion, this method, even if it requires specific equipment, is fast, reliable, highly sensitive and specific for detection of SMA carriers, which is not possible with other routine methods.

Acknowledgements

We want to thank Dr. Antonio Toscano and Dr. Carmelo Rodolico for providing blood of SMA patients and their relatives; Dr. Marco Cappelletti and Dr. Nicola Cirenei, application specialists Applied Biosystem, for their technical support in the choice of primers and probe.

References

[1] Munsat TL, Davies KE. International SMA consortium meeting. (26–28 June 1992, Bonn, Germany). Neuromuscul Disord 1992;2(5–6):423–8.
[2] Brzustowicz LM, Lehner T, Castilla LH, Penchaszadeh GK, Wilhelmsen KC, Daniels R, et al. Genetic mapping of chronic childhood-onset spinal muscular atrophy to chromosome 5q11.2–13.3. Nature 1990;344(6266):540–1.
[3] Lefebvre S, Bürglen L, Reboullet S, Clermont O, Burlet P, Viollet L, et al. Identification and characterization of a spinal muscular atrophy-determining gene. Cell 1995;80(1):155–65.
[4] Scheffer H, Cobben JM, Matthijs G, Wirth B. Best practice guidelines for molecular analysis in spinal muscular atrophy. Eur J Hum Genet 2001;9(7):484–91.
[5] Pearn J. Classification of spinal muscular atrophies. Lancet 1980;1(8174):919–22.
[6] Van der Steege G, Grootscholten PM, van der Vlies P, Draaijers TG, Osinga J, Cobben JM, et al. PCR-based DNA test to confirm clinical diagnosis of autosomal recessive spinal muscular atrophy. Lancet 1995;345(8955):985–6.
[7] Mazzei R, Conforti FL, Muglia M, Sprovieri T, Patitucci A, Magariello A, et al. A simple method for diagnosis of autosomal recessive spinal muscular atrophy by denaturing high-performance liquid chromatography. J Child Neurol 2003;18(4):269–71.
[8] Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16(3):1215.
[9] Wilke K, Duman B, Horst J. Diagnosis of haploidy and triploidy based on measurement of gene copy number by real-time PCR. Hum Mutat 2000;16(5):431–6.
[10] Aarskog NK, Vedeler CA. Real-time quantitative polymerase chain reaction. A new method that detects both the peripheral myelin protein 22 duplication in Charcot-Marie-Tooth type 1A disease and the peripheral myelin protein 22 deletion in hereditary neuropathy with liability to pressure palsies. Hum Genet 2000;107(5):494–8.
[11] Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. Genome Res 1996;6(10):986–94.
[12] Lie YS, Petropoulos CJ. Advances in quantitative PCR technology: 5' nuclease assays. Curr Opin Biotechnol 1998;9(1):43–8.
[13] Wirth B, Herz M, Wetter A, Moskau S, Hahnen E, Rudnik-Schöneborn S, et al. Quantitative analysis of survival motor neuron copies: identification of subtle SMN1 mutations in patients with spinal muscular atrophy, genotype-phenotype correlation, and implications for genetic counseling. Am J Hum Genet 1999;64(5):1340–56.
[14] Feldkötter M, Schwarzer V, Wirth R, Wienker TF, Wirth B. Quantitative analyses of SMN1 and SMN2 based on real-time lightCycler PCR: fast and highly reliable carrier testing and prediction of severity of spinal muscular atrophy. Am J Hum Genet 2002;70(2):358–68.